Transient Increase in Circulating Donor Leukocytes After Allogeneic Transfusion in Immunocompetent Recipients Compatible With Donor Cell Proliferation

By Tzong-Hae Lee, Elizabeth Donegan, Sherrill Slichter, and Michael P. Busch

Donor leukocytes in therapeutic blood components are implicated in transfusion-related complications ranging from alloimmunization to graft-versus-host disease (GVHD) to viral transmission and reactivation. To further characterize the kinetics of donor leukocyte clearance after allogeneic transfusion, we developed allele-specific polymerase chain reaction (PCR) assays directed at a single-copy Y chromosome gene and HLA class II alleles. These assays enable sensitive detection and quantitation of donor leukocytes at concentrations ranging from one cell to greater than 1,000 cells per 125 μL of recipient blood. When applied to serial samples from five consecutive orthotopic surgery patients who met study criteria, we observed 99.5% clearance of donor leukocytes over the initial 2 days posttransfusion, followed by a transient, 1-log increase in circulating donor leukocytes on days 3 to 5. This phenomenon was reproduced in a canine transfusion model, where the transient donor leukocyte expansion phase was prevented by gamma irradiation of donor blood, and was not observed after transfusions into alloimmunized dogs. We hypothesize that this transient increase in circulating allogeneic donor cells represents one arm of an in vivo mixed lymphocyte reaction, with activated donor T lymphocytes proliferating in an abortive GVHD reaction to HLA-incompatible recipient cells. Further investigation of this phenomenon should provide insight into the mechanisms involved in donor-recipient leukocyte interactions posttransfusion and the relationship of these interactions to leukocyte-induced complications.

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sequences in nucleated donor cells under conditions that minimize and control for allelic DNA competition in background recipient leukocytes. Quantitation is achieved by parallel analysis of a dilution series of donor blood (or leukocytes) into recipient blood (or leukocytes) obtained before transfusion. When this method was used to study the kinetics of donor leukocyte survival after allogeneic transfusion of immunocompetent subjects, we discovered that, after an initial clearance phase, the concentrations of circulating donor cells increased transiently in recipients.

MATERIALS AND METHODS

Subjects Studied and Transfusion Protocols

Orthopedic surgery patients. Female patients were identified from the University of California, San Francisco (UCSF) Medical Center (San Francisco, CA) Maximum Blood Order Schedule who were scheduled for an elective orthopedic surgery procedure for which at least 1 U of unmanipulated (ie, not irradiated or leukodepleted) packed RBC was likely to be transfused, and from whom autologous blood was not collected. Before selection for the study, the subjects’ charts were reviewed to determine indications for surgery and prior pregnancy and transfusion history, and to exclude medical conditions suggesting possible immunodeficiency. Fully screened male donor RBC units less than 14 days old were crossmatched with the selected recipients’ blood until a single compatible male donor RBC unit was identified. This unit was designated the index unit. All additional blood components for these patients were selected from the fully screened blood inventory based on their having been collected from female donors. One tubing segment of blood from each donor unit was retained by the transfusion service and forwarded to the testing laboratory for (1) verification of male/female origin by Y chromosome positivity, (2) HLA-DR typing by allele-specific PCR (see below), and (3) use in dilutional studies (index units) with pretransfusion recipient blood to generate the standard curve for interpretation of donor cell concentrations in posttransfusion samples. Pretransfusion and posttransfusion EDTA-anticoagulated blood samples from the patients were obtained throughout the hospital stay from the hematology laboratory after completion of routinely ordered diagnostic testing. All five patients had uneventful postoperative recoveries without reported transfusion reactions.

As no additional procedures (including phlebotomies) were performed for purposes of this study, specific informed consent was not required or obtained from recipients or donors. The study protocol was approved by the UCSF Committee on Human Research.

Canine transfusion studies. Donor blood (1 U) was obtained the day before intended transfusion from a type-compatible, 32-kg male dog by collection into an ACD quad-pack blood bag and processed into three equal packed RBC components with Nutricell additive (Cutter Laboratories, Covena, CA) according to routine blood bank procedure. Three adult female dogs were sedated (acepromazine, 0.05 mg/kg; butorphanol, 0.2 mg/kg), and 5 mL venous blood was collected into EDTA tubes as pretransfusion samples. A volume of donor blood estimated to represent \( \frac{1}{10} \) the total blood volume of the recipient was transfused intravenously. Venous blood (3 mL) was collected at 15 minutes, 2 hours, and daily posttransfusion for 1 week. Donor blood for one of the three recipient dogs was exposed to 1,500 cGy gamma irradiation before transfusion, according to standard blood bank procedure.

An additional female dog was transfused on two occasions with fresh ACD-anticoagulated whole blood from a male donor to which the recipient had been previously alloimmunized by repeated transfusions of platelet concentrates, as documented by radiolabeled platelet survival and antibody measurements. A nonimmunized female recipient dog was transfused with platelet-rich plasma from a male donor; the platelet-rich plasma was treated with 8-methoxypsoralen (300 \( \mu \)g/mL) and UV A light (340 to 400 nm; 7.5 J/cm\(^2\)) before transfusion. The canine studies were approved by the UCSF and University of Washington (UW; Seattle, WA) committees on animal research.

Cell/DNA Preparation and Processing

To quantitatively recover leukocytes from recipient blood samples, 0.5 mL of anticoagulated whole blood was subjected to RBC lysis using 0.5 mL saponin lysis buffer (0.4% saponin [ICN Biochemicals, Cleveland, OH] in 0.5% NaCl) within 12 to 24 hours of phlebotomy. After a 5-minute incubation at room temperature with intermittent mixing, the tube was centrifuged at 3,000 rpm (600g) for 5 minutes in a microcentrifuge. The leukocyte pellet was then washed twice with 1 mL PCR solution A (100 mmol/L KCl; 10 mmol/L Tris, pH 8.3; 2.5 mmol/L MgCl\(_2\); 1% Tween-20; 1% NP40; 0.4 mg/mL proteinase K) to each pellet and incubating at 60°C for 1.5 hours with vortexing every 20 minutes, followed by 95°C for 2 hours. The DNA lysates from each serial blood were then tested together in duplicate, both neat and at a 1:10 dilution of each DNA lysate, in a single PCR/hybridization/polyacrylamide gel electrophoresis (PAGE)/autoradiography run along with analysis of duplicate standard dilution series. Analysis of a 1:10 dilution of lysates is intended to detect PCR inhibitors, as well as to yield signal in the linear range of the standard curve for samples with high levels of target cells (see below). By testing the serial samples from each patient in a single run, we controlled for possible patient-specific factors (eg, variable background leukocyte concentrations) and eliminated potential between-run variables, such as slight differences in PCR reaction conditions or \(^{32}P\) specific activity.

Development and Standardization of QSS-PCR

Based on published sequence data, we designed primers that would selectively amplify single-copy gene alleles unique to minor target cell populations to be studied (ie, DR1, DR2, and SRY). For each assay, four candidate primer sequences (see below) were synthesized and evaluated in different combinations. Different amplification reaction mixtures and temperature cycling programs were also evaluated to identify optimal amplification conditions for each primer pair combination. Conditions were first optimized using serial dilutions of DR1-, DR2-, or Y chromosome-positive cells prepared in parallel into buffer and into suspensions of 1 \( \times 10^8 \) DR1-, DR2-, and Y chromosome-negative cells, respectively. For each system, primer combinations and amplification conditions were identified that enabled detection of as few as one to five target leukocytes in a sample containing \( 10^8 \) allogeneic recipient leukocytes, with accurate quantitation over approximately 3-log concentrations of target cells.

A series of replicate analyses of spiked whole blood samples was then performed to document accuracy and precision of the assay over the quantitative range to be investigated. Three different donor whole blood samples (positive for the target alleles) with known leukocyte concentrations were diluted fourfold, and the serial dilutions were spiked into three different recipient whole blood samples (negative for the target alleles). Each dilution was then processed through the assay system in replicates of five. Means, standard deviations, and coefficients of variation at each spike level were determined.
PCR Primers and Conditions

HLA DR1 and DR2 allele-specific PCR. A 91-bp region of DRB1 gene was amplified using DR1 allele-specific primer pairs (5' CTGTGCGACCTTAAGTTGGAATG 3') and DR2 (5' GGAGACCTCCTGAGTTATA-GATGCA 3'). A 258-bp region of DR2 was amplified using allele-specific primers DR2A (5' CTGTGCGACCTTAAGGAGGAGT 3') and DR2B (5' CGCGCTGACTGGAAGCTCT 3'). The PCR reaction mixture for both DR1 and DR2 primers consisted of 100 mmol/L KCl, 20 mmol/L Tris HCl pH 8.3, 2.5 mmol/L MgCl2, 0.02% gelatin, 2 pmol/μL of each primer, 1 mmol/L dNTPs, and 0.12 U/μL Thermalse (IBI, New Haven, CT). DNA lysate (25 μL; containing cells derived from 125 mL of whole blood) was added to 50 μL of PCR reaction mixture, followed by 35 cycles in an MJR thermocycler (MJ Research, Inc, Watertown, MA). For DR1, each cycle consisted of 25 seconds at 95°C, 1 minute at 56°C, and 1.5 minutes at 72°C. For DR2, each cycle consisted of 25 seconds at 95°C, followed by 2 minutes at 72°C.

Y chromosome sequence-specific PCR. A 148-bp region of the sex-determining region of the human Y chromosome (SRY) was amplified using allele-specific primer pairs (5' GAGCAAGATCTCATC - CTTGCTGGAAAG 3') and (5' CTGGTGCGAGCTTAAAGGGAGT 3'). The PCR reaction mixture consisted of 100 mmol/L KCl, 20 mmol/L Tris HCl pH 8.3, 2.5 mmol/L MgCl2, 0.02% gelatin, 1 pmol/μL of each primer, and 0.04 U/μL Thermalse. DNA lysate (25 μL) was added to 50 μL of PCR reaction mixture, followed by amplification for 30 cycles consisting of 30 seconds at 95°C, 2 minutes at 70°C. For DR2, each cycle consisted of 25 seconds at 95°C, followed by 2 minutes at 72°C.

Detection and Quantitation

A gelatin, 2 pmol/μL of each primer, 1 mmoVL L KCl, 20 %oVL Tris HCI pH 8.3, 2.5 ~IIIOVL MgCI2, 0.02% gelatin, 2 pmol/μL of each primer, and 0.12 U/μL Thermalse (IBI, New Haven, CT). DNA lysate (25 μL; containing cells derived from 125 mL of whole blood) was added to 50 μL of PCR reaction mixture, followed by 35 cycles in an MJR thermocycler (MJ Research, Inc, Watertown, MA). For DR1, each cycle consisted of 25 seconds at 95°C, 1 minute at 56°C, and 1.5 minutes at 72°C. For DR2, each cycle consisted of 25 seconds at 95°C, followed by 2 minutes at 72°C.

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Probe Sequences and Hybridization Conditions

Specific amplified products were detected by oligonucleotide liquid hybridization using 32P-labeled probes: DR1B (5' GGTCGCGTGTGCGAGAAG 3') for HLA DR1 gene, DR2B (5' TTCCGTGGACAGATCTTC 3') for HLA DR2 gene, and SB (5' GAGGCGCAAGATGGTCTACAG 3') for human and canine Y chromosomes. The probes were end-labeled at 37°C for 1 hour in a 40-μL solution consisting of 7 mmol/L Tris HCl pH 7.6, 10 mmol/L MgCl2, 15 mmol/L diethanol, 10 U T4 polynucleotide kinase (New England Biologicals, Cambridge, MA), 40 μCi 32P-adenosine triphosphate (32P-ATP; Dupont, Wilmington, DE) and 30 pmol probes. After incubation, the volume was adjusted to 400 μL by addition of 0.75 mmol/L NaCl and 10 mmol/L EDTA solution. For hybridization, 10 μL of this probe mix was added to 30 μL of postamplification specimen and incubated for 5 minutes at 59°C after a 5-minute denaturation at 95°C.

Detection and Quantitation

To each hybridized sample, 10 μL of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, and 30% glycerol) was added, and this mixture was subjected to 6% PAGE at 12.5 V/cm. After running, the gel was exposed to XAR-5 autoradiographic film (Kodak, Rochester, NY) with enhancing screen for 12.5 V/cm. After running, the gel was exposed to XAR-5 autoradiographic film (Kodak, Rochester, NY) with enhancing screen for 12.5 V/cm. After running, the gel was exposed to XAR-5 autoradiographic film (Kodak, Rochester, NY) with enhancing screen for 12.5 V/cm. After running, the gel was exposed to XAR-5 autoradiographic film (Kodak, Rochester, NY) with enhancing screen for 12.5 V/cm.
**Donor Leukocyte Survival Studies in Humans**

Our first donor leukocyte survival study involved tracing leukocytes in 1 U of packed RBC from a male, DR1-positive donor after transfusion (3 days postcollection) into a 64-year-old, DR1-negative woman undergoing total hip replacement. The number of infused donor leukocytes was calculated based on weight of the blood bag pretransfusion and post-clearance. Blood specimens collected at serial time points posttransfusion were then evaluated using QSS-PCR directed at Y chromosome and DR1 target genes to determine posttransfusion donor leukocyte survival, expressed as the concentration of donor cells per milliliter of recipient peripheral blood.

As seen in Fig 1, a progressive decline in circulating donor leukocytes was observed over the first 3 days posttransfusion, with a nadir of approximately 10 donor cells per milliliter of blood on day 3 in patient 1. This was followed by an approximately 10-fold increase in the concentration of circulating donor leukocytes at 4 days posttransfusion, followed by a secondary clearance of donor cells on days 5 to 7. In this patient, an additional RBC unit from a DR1-positive, female donor was infused on the afternoon of day 5. Note that PCR signal attributable to transfused leukocytes in this unit was detected on day 6 using the DR1 system (Fig 1B) but, because the donor was female, not the Y chromosome system (Fig 1A).

Figure 2 shows QSS-PCR results using the Y chromosome system for four additional female orthopedic surgery patients, each transfused with single units of male donor RBC. These patients ranged in age from 17 to 47 years, and received index RBC units that had been stored at 4°C for from 4 to 14 days before transfusion. Although the posttransfusion sampling time points for several of these patients were not as complete as for the patient shown in Fig 1, all except patient 5 showed results consistent with a decline in circulating male donor leukocytes over the first 2 to 3 days posttransfusion, followed by an increase in the concentration of donor cells on days 3 through 5.

**Donor Leukocyte Survival in Canine Transfusion Model**

The kinetics of donor leukocyte clearance after transfusion were further studied in the canine transfusion model. In initial studies, two female recipients were transfused with 80 mL of 1-day-old RBC component derived from a single male donor dog; a third female dog was transfused with 70 mL of gamma-irradiated (1,500 cGy) RBC from the same male donor dog. Samples collected immediately posttransfusion and then daily for 7 days were tested by QSS-PCR analysis directed at canine Y chromosome-specific sequences. For the recipients of nonirradiated blood, the kinetics of initial clearance, followed by a transient increase in circulating male donor leukocytes, were similar to that was observed in humans (Fig 3A). In contrast, in the recipient of irradiated RBC, the transient increase in circulating donor leukocytes at days 3 to 5 posttransfusion was not observed (Fig 3B).

An additional female dog, which had been previously alloimmunized to a male donor as determined by platelet antibody studies and rapid clearance of radiolabeled platelets, was transfused on two occasions with fresh, nonirradiated whole blood from the donor dog. This alloimmunized dog showed accelerated donor leukocyte clearance (ie, no male cells detectable by 24 hours posttransfusion) and no evidence of subsequent increase in circulating donor leukocytes. Finally, a nonalloimmunized female recipient was transfused on two occasions with platelet-rich plasma that was treated with 8-methoxypsoralin and UV-A light before transfusion. The results for both transfusions were similar to those after transfusion of irradiated blood, with initial leukocyte clearance by day 3 and no detectable subsequent increase in circulating donor cells (data not shown).

**DISCUSSION**

Although previous investigators have reported detection of donor leukocytes in the circulation of immunocompetent recipients during the first week posttransfusion, the kinetics of clearance have not been previously characterized. In the present study, we used a QSS-PCR assay directed at Y chromosome and HLA sequences to quantitatively study donor leukocyte survival after allogeneic transfusion. In both humans and dogs, we observed a progressive decline in circulating donor leukocytes over the first several days posttransfusion, followed by an approximately 10-fold increase in the concentration of donor leukocytes 3 to 6 days posttransfusion. The increase in circulating donor leukocytes was not detected in female dogs transfused with gamma-irradiated or photosensitized (8-methoxypsoralin and UV-A) blood, which suggests that this leukocyte expansion phase may be due to proliferation of donor leukocytes (probably lymphocytes), rather than recirculation of cells that were sequestered in the spleen or reticuloendothelial system.

Thus, as first hypothesized by Schechter et al in 1972 and later reviewed, transfusion of blood containing viable donor leukocytes may routinely lead to a two-way proliferative reaction, consisting of an attempted graft-versus-host reaction by donor cells and allograft rejection by recipient cells. The timing of the initial increase in circulating donor leukocytes observed in our studies (days 3 to 5) is consistent with the 8- to 12-day delay between transfusion and clinical presentation of TA-GVHD, assuming that a lag-time of 3 to 7 days exists between earliest detection of increasing donor leukocytes by our assay and development of the levels of effector T cells necessary to induce clinical GVHD. In immunocompetent recipients such as those we studied, the donor leukocyte expansion phase is presumably aborted by an effective recipient immune response against the donor cells. In contrast, in immunosuppressed recipients or in homozygous recipients of haplotype-mismatched transfusions, donor leukocyte expansion may progress unchecked, leading to clinical GVHD. Further studies will be required to establish that the transient donor leukocyte expansion phase observed in our study represents an allotypic prolifera-
Fig 1. Results of tracking male, DR1-positive donor leukocytes from 1 U of packed RBC transfused into a 64-year-old, DR1-negative woman immediately after total hip replacement. Representative PCR autoradiographic results are presented above the corresponding time point on the survival graphs. Above the autoradiograph lanes, the analysis of undiluted (I) and a 1:10 dilution (II) of each DNA lysate are indicated. Transfusion of male DR1-positive unit is indicated by arrow a. No SRY- or DR1-positive cells were detected in the pretransfusion samples. The initial concentration of donor leukocytes immediately after transfusion was calculated based on volume and concentration of donor leukocytes in the transfusion and the recipient’s blood volume (dashed lines and open circles). Blood specimens collected at serial time points posttransfusion (indicated as solid circles) were evaluated using QSS-PCR directed at Y chromosome SRY (A) and DR1 (B) target genes. Note progressive decline in circulating donor leukocytes over the first 3 days posttransfusion to a nadir of approximately 10 donor cells per milliliter, followed by an approximately 10-fold increase in circulating donor leukocytes at 4 days posttransfusion. Note also that a unit from a DR1-positive, female donor transfused late on day 5 was detected by the DR1 system but not the Y chromosome system (transfusion indicated by arrow b).
tion of donor T cells consistent with an abortive GVHD reaction, including determination of the immunophenotype(s) and immunologic specificity of donor leukocytes isolated from recipient blood during this expansion phase.39

In addition to a possible relationship to GVHD, the transient donor leukocyte expansion phase observed in our study may have relevance to several other complications of blood transfusions. The absence of detection of a donor leukocyte recirculation phase in a dog that was alloimmunized to its donor suggests a possible relationship between the donor leukocyte expansion phenomenon and alloimmunization. The fact that gamma-irradiated blood can induce a platelet-refractory state clearly indicates that mitotic expansion of donor cells is not a prerequisite to alloimmunization. However, there may be a continuum in alloimmunogenic potential, ranging from fresh, viable leukocytes to intact but nonviable leukocytes to leukocyte membrane fragments.41 Future studies should investigate the relationship between the kinetics of donor leukocyte clearance and recipient immune activation posttransfusion, with development of alloimmunization, as well as the effect of manipulations intended to reduce alloimmunization (e.g., leukodepletion and photochemical treatments) on these phenomena.

Lang first proposed a relationship between mitotic proliferation of donor and recipient leukocytes after transfusion and induction of latent viral infections in donor and recipient leukocytes. Subsequent animal14 and human46,47 studies have indicated that viable, allogeneic donor leukocytes are instrumental in inducing both primary and reactivation cytomegalovirus (CMV) infections after transfusions. Recent in vitro48 and clinical studies49,50 suggest that transfusion of donor leukocytes may reactivate human immunodeficiency virus (HIV) and may lead to increased opportunistic infections (particularly CMV) and accelerated mortality in HIV-infected patients. A multicenter study of transfusion-induced viral reactivation and GVHD in HIV-infected patients will begin clinical activity in early 1995 (E. Sloand, National Heart, Lung and Blood Institute). In this study, leukocyte survival kinetics will be monitored using quantitative PCR techniques similar to those used in the present study, and the results will be correlated with viral reactivation and clinical outcome.
DONOR LEUKOCYTE RECIRCULATION

In conclusion, our results indicate that the concentration of donor leukocytes in recipient blood increases transiently 3 to 5 days posttransfusion. We hypothesize that this transient increase in circulating donor leukocytes represents one arm of an in vivo mixed lymphocyte reaction, with activated donor T lymphocytes proliferating in an abortive GVHD reaction to HLA-incompatible recipient cells. Further investigation of this phenomenon should provide insight into the mechanisms involved in donor-recipient leukocyte interactions posttransfusion and the relationship of these interactions to leukocyte-induced complications.

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Transient increase in circulating donor leukocytes after allogeneic transfusions in immunocompetent recipients compatible with donor cell proliferation

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